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FIELD AND LABORATORY STUDIES OF *GLUGEA HERTWIGI* (MICROSPORIDA) IN THE RAINBOW SMELT *OSMERUS MORDAX* [1]

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Glugea hertwigi-induced microsporidosis is a disease of the smelt, *Osmerus mordax*. *O. mordax* is an anadromous species that has been successfully introduced to temperate freshwater areas. Haley (1957) has provided some evidence which indicates *G. hertwigi* was in part responsible for the decline of the smelt fishery in the Atlantic. The incidence of *Glugea* infection reaches a seasonal peak of nearly 90% in juvenile Lake Erie smelt each summer and fall; Nepszy, Budd, and Dechtiar (1978) estimate vast economic losses in the smelt fishery in the Great Lakes due to mortality of infected juveniles.

G. hertwigi infections typically localize as parasite colonies in the submucosal layer of the intestine. Mortaility of the smelt host is believed to occur by starvation; in addition, infected fish have navigation problems, are more susceptible to predation, and less able to recover from environmental stress (Legault and Delisle, 1967; Nepszy and Dechtiar, 1972). Spawning female smelt in Lake Erie characteristically have numerous *G. hertwigi* cysts in the ovaries as well as along the intestine. Chen and Power (1972) reported a 42% decrease in fecundity of *Glugea* infected females. The microsporidan *Plistophora ovariae* infecting the golden shiner *Notemigonens crysoleucas* is an example of transovarian parasite transmission (Summerfelt and Warner, 1970).

G. hertwigi is an obligate intracellular parasite completing its life cycle within a single host cell. After ingestion by a smelt, the infective spore is believed to discharge a polar filament with a velocity suitable for penetrating the intestinal mucosal cell layer (Ishihara, 1968; Weidner, 1972, 1976). The vegetative stages of the parasite do not cause host cell degeneration but stimulate hypertrophy and abnormal development into a "xenoma" (Weissenberg, 1968, 1976; Weidner, 1974). "Xenoma" refers to the unique association between an hypertrophied host cell and developing intracellular parasites (Weissenberg, 1968, 1976). The host-cell component is induced to undergo extensive growth during vegetative development (schizogony) by the parasite. Eventually the parasite differentiates into mature spores (sporogony) which fill the central region of the xenoma. By this stage, a combination of host animal response and parasite growth transform the xenoma into a thick-walled "Glugea-cyst" filled with innumerable spores (Sprague and Vernick, 1968; Weissenberg, 1968, 1976; Weidner, 1973, 1976). *G. hertwigi* cysts range from 0.4 to 5.0 mm in diameter; heavy smelt infections

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number over 200 xenomas per host (Legault and Delisle, 1967; Nepszy and Dechtiar, 1972).

McVicar (1975), Olson (1976) and Weissenberg (1968) transmitted *Glugea* species to fish held in the laboratory. Weidner (1973) and Stunkard and Lux (1965) suggested that invertebrate filter-feeders may serve as natural vectors or transport hosts for fish microsporidans. Olson (1976) determined a low level of *Glugea stephani* infection occurred by ingestion of spores directly. McVicar (1975) transmitted *G. stephani* through spore-carrying vectors and by injection of spores into the peritoneal cavity of adult fish. Weissenberg (1968) did not determine whether *G. anomala* was inititated by a spore-carrying vector or by direct ingestion of the spores.

We thought it would be of interest to examine *G. hertwigi* growth and multiplication in ovaries of spawning female smelt; follow transmission of infections; and examine the microstructure of early xenoma growth.

Materials and Methods

Adult smelt

Spawning female smelt were collected from Wheatley, Ontario, on Lake Erie and from the Jones River, near Plymouth, Cape Cod, Massachusetts. A total of 150 fish from each location were examined internally for the presence of *Glugea hertwigi* cysts. The intestine and ovaries from infected and non-infected fish were excised, cut into small pieces and fixed in pH 7.4 phosphate buffered glutaraldehyde overnight at 4° C. After several buffer rinses, the material was post-fixed in phosphate buffered 2% osmium tetroxide for 2 hr at 4° C, dehydrated in ethanol and embedded in Epon. One micron sections were cut on a Dupont-Sorvall MT-2B microtome and stained with 1% toluidine blue. Parasite cysts were removed from adult smelt, homogenized, pelleted in pH 7.4 phosphate buffer, stored at 4° C and used for the transmission experiments.

Transmission experiments

Eggs and milt were stripped from spawning smelt, mixed 1:5 respectively and kept in a well-aerated nylon mesh cone. Naturally fertilized eggs were also collected from the river-bed. Anticipating large mortalities, a non-infected smelt population was located in Long Pond, Cape Cod, Massachusetts. Several years' examination of smelt from this pond had indicated they were completely free of *G. hertwigi* infection. Young smelt (20–25 mm) in Long Pond were attracted at night to a strong light at the surface and collected by hand-net. All fish were maintained at the National Marine Fisheries Service Aquarium, Woods Hole, in 20° C filtered fresh water taken from the Jones River well above the spawning sites. All fish were maintained on a diet of phyto- and zooplankton seined from Long Pond. The following methods of parasite transmission were attempted.

Experiment 1. Laboratory reared and collected smelt were exposed to a suspension of *G. hertwigi* spores placed in the tanks. The water was well aerated but not filtered for the following 48 hr.

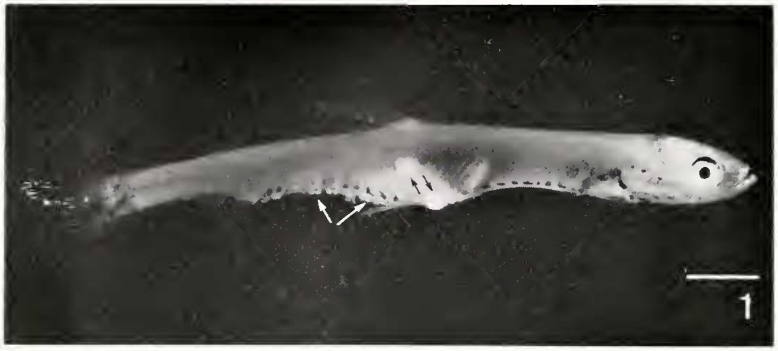

FIGURE 1. Young specimen of the smelt, *Osmerus mordax*, experimentally infected with *Glugea hertwigi*. The yearly incidence of natural infections nears 90% in juveniles with consequent vast mortalities. Seven days post infection, xenomas (white arrows) develop proximate to larger 4-week-old "Glugea cysts" (black arrows). Bar represents 2.5 mm.

Experiment 2. Laboratory reared smelt (10 mm, 6 weeks post spawning) were fed spore-carrying zooplankton (cladocerans and copepods) on 2 consecutive days. The plankton was first exposed to a spore suspension for 30 min, washed once with water, examined to ensure the presence of spores in their digestive tracts and then fed to the smelt.

Experiment 3. Collected smelt (15 mm, approximately 8 weeks post spawning) were fed spore-carrying plankton as above.

Experiment 4. Smelt from Experiment 1, 4 weeks after exposure to a spore suspension, were fed spore-carrying plankton as above. As a control, a number of laboratory reared and collected smelt were maintained unexposed to spores.

Development of xenomas

Young smelt from all tanks were observed and photographed with a Wild M–4 Makroskope 5 days, 1, 2 and 3 weeks after spore feeding. Intestinal tissue from experimental and control smelt were prepared for microscopy as outlined above.

TABLE I

Experiment number	Source of smelt	Sample size	Method of exposure	Period of incubation	Number of smelt infected	
					Light < 5 (per host)	Heavy > 50 (per host)
1	mixture of lab reared and collected	20	spore suspension	28 days	8	0
2	lab reared	5	spore-carrying plankton	7 days	0	5
3	collected	14	spore-carrying plankton	7 days	0	14
4*	mixture	8	spore-carrying plankton	7 days	0	8

* Smelt from Experiment 1, carrying light infections 28 days after exposure to the spore suspension.

Results

Adult smelt

In spawning females *Gluga hertwigi* cysts were found in the ovaries of more than 50% of the infected fish in Lake Erie and in 25% of infected fish in the Jones River. The ovaries of Lake Erie fish were heavily infected whereas those of the Jones River were lightly infected. Sections of infected ovaries showed the parasite was isolated from the scattered ova by the cyst wall. No free spores or developing stages of *G. hertwigi* were observed outside the cysts, in ova or in ovarian tissue.

Parasite transmission

The results of the experiments are summarized in Table I. Positive results were obtained from both methods of spore transmission; however, the intensity of infection and its effect differed.

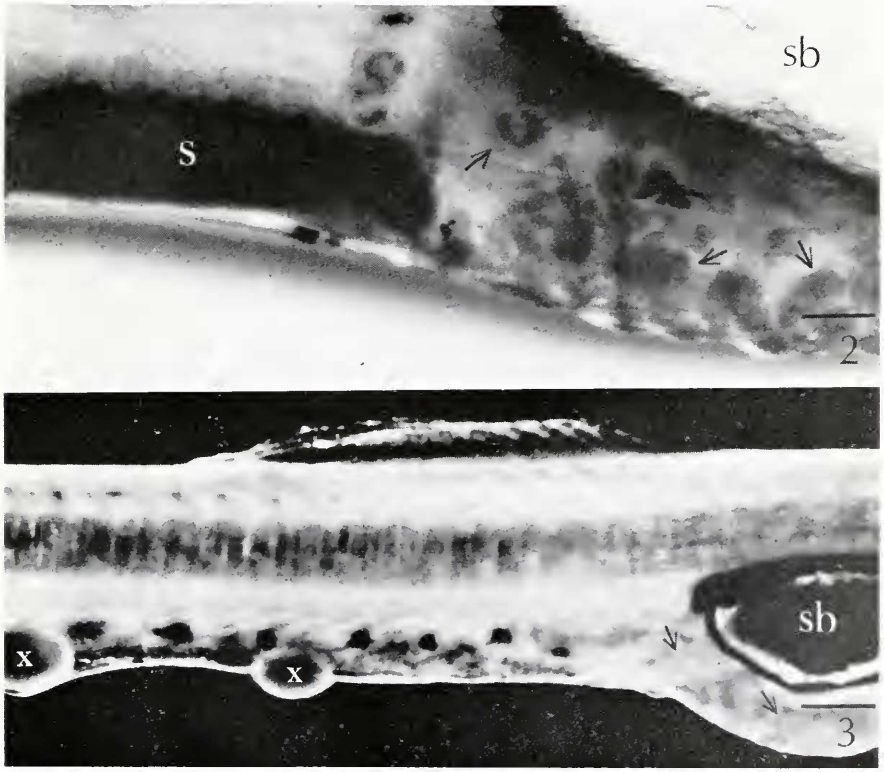

FIGURE 2. Young smelt, 12 days after feeding on spore-carrying plankton. Numerous xenomas (arrows) begin just behind the stomach and continue along the gut to the vent. S, stomach; sb, swimbladder. Bar represents 180 μm.

FIGURE 3. Young smelt previously infected after exposure to a spore suspension developed numerous new xenomas (arrows) along the intestine when fed spore-carrying plankton. X, xenomas from exposure to spores directly. Bar represents 750 μm.

Experiment 1. Laboratory-reared and collected fish were exposed to a suspension of *G. hertwigi* spores. Four weeks after exposure, 40% of the smelt exhibited one or two *Glugea* cysts in the posterior region of the intestine. These cysts were uniformly dense and protruded from the submucosa distending the peritoneal cavity. The fish fed continually throughout the experimental period. Several fish died during the period, were examined and one was found infected with a single *Glugea* cyst.

Experiment 2. Laboratory-reared fish were exposed to spore-carrying plankton. One week after exposure all fish exhibited numerous small xenomas beginning just behind the stomach and continuing along the entire length of the intestine to the vent (Fig. 2). Gradually the fish stopped feeding, had difficulty swimming and all died by 16 days after exposure to the spores.

Experiment 3. Collected smelt were exposed to spore-carrying plankton. The results were similar to those in Experiment 2; however, these fish stopped feeding and died at about 25 days after exposure to the parasite.

Experiment 4. Smelt from Experiment 1, 4 weeks after being exposed to a spore suspension, were fed spore-carrying plankton. One week after feeding, all fish exhibited numerous small xenomas along the intestine as well as the large posterior xenomas (Fig. 3). All fish died within 2 weeks after exposure to the spore-carrying vectors. Controls were examined periodically and found free of infection.

Parasite development

Parasite growth was rapid at 20° C. Examination of the intestine from a heavy infection (Experiments 2, 3, and 4) showed the extensive tissue involvement. Infections protruded to the serosa and were easily dissected away intact (Fig. 4). Extensive host cell hypertrophy was the obvious feature of sectioned material (Fig. 5). Xenomas ranged from 20 to 50 μm in diameter. Smaller xenomas contained one or two greatly enlarged host cell nuclei; whereas the larger xenomas

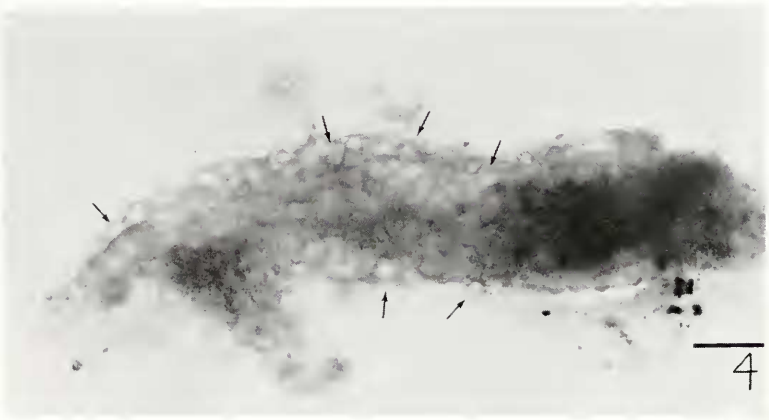

FIGURE 4. Whole intestine 1 week after exposure to spore-carrying plankton. Xenomas (arrows) protrude to the serosa and are easily dissected away intact. Bar represents 80 μm.

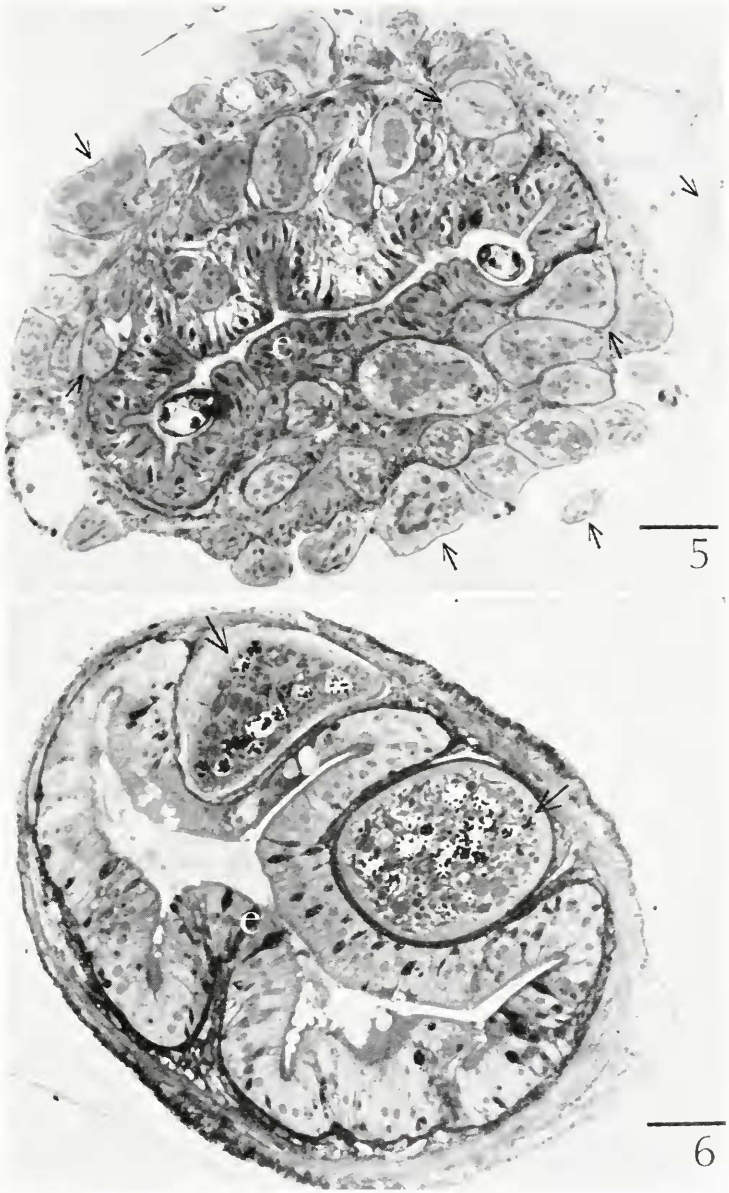

FIGURE 5. Cross section of intestine similar to that shown in Figure 4. At least 40 xenomas (arrows) 20 to 50 μm in diameter develop within the submucosa and protrude from the mucosa. Infected host cells hypertrophy and contain early schizont stages of the parasite. e, epithelium. One micron Epon section; 1% toluidine blue stain; bar represents 50 μm.

FIGURE 6. Cross section of intestine from lightly infected smelt 2 weeks after feeding on a spore suspension. Progressive hypertrophy increased the xenomas to 100 to 125 μm in diameter. Xenomas develop in the submucosal layer causing mechanical distension of the epithelium (e). Sporogony stages and free dense spores fill the central region of the xenoma. One micron Epon section; 1% toluidine blue stain; bar represents 50 μm.

were multinucleated with many nuclei lobed or branched, indicating nuclear division. At 1 week the parasite formed schizont colonies peripheral to the host cell nuclei. After 2 weeks of growth there was considerable increase in host cell hypertrophy and *Glugea* maturation to spores. Xenomas ranged from 100 to 125 μm in diameter with host cell nuclei, cytoplasmic components and *G. hertwigi* schizonts particularly obvious in the peripheral region of the xenomas; sporoblastic stages of the parasite were common in the central region (Fig. 5).

Discussion

Transovarian parasite transmission is known from a number of microsporidan species (Kudo, 1966). Recently, Summerfelt and Warner (1970) demonstrated a *Plistophora ovariae* infection in viable eggs of the golden shiner, *Notemigonus crysoleucas*. Although the ovaries of spawning female smelt were often loaded with *G. hertwigi* parasites, our thorough examinations indicate *G. hertwigi* are not present in germinative or egg cells. Previously, there has been limited success in experimental peroral transmission of fish microsporidans (Delisle, 1969; McVicar, 1975; Summerfelt and Warner, 1970; Stunkard and Lux, 1965). However, Olson (1976) successfully transmitted *G. stephani* to the English sole, *Parophyrys vetulus* in water temperatures above 15° C. Several authors are convinced that transport vectors are necessary to concentrate *Glugea* spores for natural transmission of certain fish microsporidosis (Haley, 1957; Putz and McLaughlin, 1970; Stunkard and Lux, 1965). Small filter-feeding animals may serve as transport hosts and in addition, may stimulate the spores to hatch and infect the fish. Weidner (unpublished observations) has observed such a phenomenon with *G. stephani* in the winter flounder, *Pseudopleuronectes americanus*. In this study, transmission of *G. hertwigi* to both laboratory-reared and collected smelt was successful at 20° C, either by direct spore consumption or ingestion of spore-carrying vectors; however, a major magnitude of difference exists in the intensity between direct spore and vector transmitted infections. Vector transmission produced massive infections along the entire intestine in all test subjects.

Smelt are selective predators, taking cyclopoid and calanoid copepods and several species of cladocerans as their first food (Reif and Tappa, 1966; Siefert, 1972). Presumably, the natural *G. hertwigi* infection occurs through the ingestion of spore-carrying filter feeders by very young smelt. Release of spores from infected adult smelt occurs via two routes. Scarborough (unpublished observations) has observed the expulsion of parasite xenomas from ovaries during spawning. In this manner, female adult smelt may concentrate *G. hertwigi* spores in the immediate vicinity of developing young. Further, infected smelt carrion were seen being preyed upon by small crustaceans in the nursery areas after spawning. Nepszy and Dechtiar (1972) found that heavily infected adult smelt were unable to recover from spawning stress; mass mortalities in the spawning grounds consisted of infected adults.

Massive infections of *G. hertwigi* consequent to ingestion of spore vectors are fatal to both collected and laboratory reared smelt. Mechanical distention of the intestinal tissue and starvation are thought to be the cause of death. *Osmerus mordax* is a difficult species to raise in the laboratory and the minimal condition

of the reared fish probably precluded a greater susceptibility to the effects of multiple infections. Young smelt tolerate light infections for at least several weeks and likely carry them into adulthood. Nepszy and Dechtiar (1972) found that *G. hertwigi* colonies can remain in smelt for much of the host's life, and stress will significantly increase the mortality rate in these fish over uninfected smelt.

It is well documented that the life cycle of microsporidans begins with injection of the sporoplasm through a spore tube into the host (Vavra, 1976; Weidner, 1972). It is assumed that the discharging tube of *G. hertwigi* spores penetrates through the gut basal lamina delivering the parasite into submucosa cells.

Whether transmitted directly by spores or via spore-carrying vectors, development of *G. hertwigi* was identical, and paralleled that described for other *Glugea* species (Sprague and Vernick, 1968; Weissenberg, 1968, 1976). Changes in macroscopic appearance of xenomas correlated with microscopic examinations of the sectioned material. While the host cell component of the xenoma remains viable, the parasite develops numerous schizonts within the peripheral cytoplasm. The host cytoplasm and nucleus hypertrophy in apparent response to the parasite's presence; subsequently, the nucleus undergoes an amitotic budding. As the xenoma size increases, a cellular capsule delimits the xenoma from the surrounding tissue; this capsule becomes enveloped by host connective tissue layers. While maturation of the parasite progresses through sporogony, host cell components begin to degenerate and spores fill the interior. The cyst stage consists of spores and scattered vestiges of host cell components surrounded by a wall.

Although Weissenberg (1976) believed the host cell to be a presumptive macrophage, the cell type which can support *G. hertwigi* remains undetermined. Our observations indicate massive infections will produce xenomas easily separated from the intestine. Xenomas were observed associated with various visceral organs, including cells below the peritoneal lining. It is not known how *G. hertwigi* enters ovarian tissue; presumably, initially infected host cells enter the blood stream and are delivered to favorable environs for growth, such as the highly vascularized ovaries. *Nosema michaelis,* a microsporidan infecting the blue crab, *Callenectes sapidus,* undergoes vegetative growth in the gut wall and these cells subsequently circulate to muscle tissue for continued development (Weidner, 1972).

This particular host-parasite association likely will be of some use in the study of xenomas. The transparency of young smelt lends itself to *in vivo* study of drug effects on parasite infection and xenoma development. Tissue culture techniques may now be carried out on isolated xenomas since these larger tumor cells are readily detectable in young smelt and easily removed aseptically from the gut serosa.

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Summary

Glugea hertwigi-induced microsporidosis is a disease of the smelt *Osmerus mordax*. The yearly incidence of infection reaches over 50% in adult smelt and as

high as 90% in juveniles. Primary infections localize as large intracellular colonies in submucosal cells of the digestive tract. Field observations indicate the ovaries of spawning females are the secondary site of infection. *G. hertwigi* was successfully transmitted to both laboratory-reared and collected young smelt at 20° C by small filter-feeding vectors and by direct ingestion of spores. Infections transmitted by spore-carrying vectors numbered hundreds per animal, and were visible along the intestine one week after feeding. Large parasitized host-cells (xenomas) extended from the intestinal serosa and were easily recovered. *G. hertwigi* infections acquired by direct spore feeding numbered one or two per animal; these fish have the capacity to develop many new infections by feeding on spore-carrying vectors. Microscopic study revealed that *G. hertwigi* development was indeed within a single greatly hypertrophied host cell. After 1 week of growth, 20 to 50-μm xenomas contained a few enlarged host nuclei and vegetative *G. hertwigi;* after 2 weeks, the xenomas measured 100 to 125 μm, exhibited multiple host nuclei and numerous *G. hertwigi* sporoblasts and spores.

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